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K-*ras* mutations in lung carcinomas from nonsmoking women exposed to unvented coal smoke in China[1]

Phouthone Keohavong[a,c,*], Qin Lan[d], Wei-Min Gao[a], David M. DeMarini[f], Marc J. Mass[f], Xue-Ming Li[e], Barbara C. Roop[f], Joel Weissfeld[b,c], Defa Tian[d], Judy L. Mumford[f]

[a] *Department of Environmental and Occupational Health, University of Pittsburgh, 3343 Forbes Avenue, Pittsburgh, PA 15260, USA*
[b] *Department of Epidemiology, University of Pittsburgh, Pittsburgh, PA 15213, USA*
[c] *The Lung Cancer Basic Research Program of the University of Pittsburgh Cancer Institute, University of Pittsburgh, Pittsburgh, PA 15213, USA*
[d] *Center for Environmental Medicine and Lung Biology, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA*
[e] *Institute of Environmental Health and Engineering, Beijing, China*
[f] *National Health and Environmental Effects Laboratory, US Environmental Protection Agency, Research Triangle Park, NC 27711, USA*

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Summary Lung cancer mortality rate in nonsmoking women in Xuan Wei (XW) County is the highest in China. The XW lung cancer rate is associated with exposure to coal smoke, containing high concentrations of polycyclic aromatic hydrocarbons (PAHs), in unvented homes. Here we investigated codon 12 K-*ras* mutations in lung tumors or sputum samples from 102 XW lung cancer patients (41 nonsmoking women and 61 smoking men). In addition, we analyzed specimens from 50 lung cancer patients (14 nonsmoking women, 33 smoking men and three nonsmoking men), from Beijing and Henan (B&H), where natural gas is the main domestic fuel. K-*ras* mutations were found in nine women (21.9%) and 14 men (22.9%) from XW, with G to T transversions accounting for 66.7 and 85.7%, respectively. Among B&H patients, one woman (7.1%) and six men (16.7%) had K-*ras* mutations, with G to T transversions accounting for 66.7% of the mutations in the men. Therefore, the frequency and type of K-*ras* mutations in XW nonsmoking women are similar to those of K-*ras* mutations found in both XW and B&H smoking men. On the other hand, the mutation frequency in XW women is higher than, although not statistically significant from, that in the B&H nonsmoking women ($P = 0.28$, two-sided Fisher's Exact Test). These results suggest an

*Corresponding author. Tel.: +1-412-383-2087; fax: +1-412-383-2123.
E-mail address: pho1@pitt.edu (P. Keohavong).

[1] We dedicate this work to the late Dr Marc Mass who passed away during the preparation of this manuscript.

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association between exposure to coal smoke and the increased K-*ras* mutation frequency in XW nonsmoking female lung cancer patients. They also suggest that the mutagens and/or mechanisms of mutations in these nonsmoking women are similar to those responsible for K-*ras* mutations in cigarette smoking lung cancer patients, which are probably induced largely by chemicals such as PAHs.

1. Introduction

Lung cancer remains the most common cause of death from cancer worldwide with over 900 000 death per year. In women, lung cancer causes 230 000 deaths annually [1]. In some regions, the lung cancer rate is particularly high. In Xuan Wei County (XW), located in Yunnan Province, China, lung cancer rate is 5-fold greater than the Chinese national average. Particularly, in some communes of that region, the lung cancer rates reach 24-fold of the Chinese national average [2]. While tobacco smoke exposure is a well-established factor for lung cancer risk, the high lung cancer mortality rate observed in XW women cannot be attributed to tobacco smoke or occupational exposure. Women in this region, who are mostly nonsmokers (smoking rate $< 1\%$), have the highest lung cancer rate in China, which is similar to the lung cancer rate in men, who are mostly smokers. This suggests that some other factors, particularly domestic ones, are responsible for the high lung cancer rate observed in XW [2]. Household fuel surveys indicate that lung cancer mortality rate in XW was highly correlated with ''smoky coal'' used for domestic combustion in this region for generations [2]. Smoky coal is a low sulfur (0.2%) medium-volatile bituminous coal used for cooking and heating in XW homes without chimneys. Characterization of indoor air from homes using smoky coal showed that XW residents were exposed to high concentrations of submicron particles that contain mostly organic matter, including a high concentration of polycyclic aromatic hydrocarbons (PAHs) which are mutagenic and carcinogenic [3]. These results point to a strong etiologic link between exposure to smoky coal combustion and the high lung cancer rate in women in XW.

Mutations in the K-*ras* proto-oncogene are frequently identified in many human cancers [4]. In lung cancer, K-*ras* mutations have been extensively studied, but mostly in smokers. These mutations are found in 15–35% of adenocarcinoma of the lung, with 80–90% of them occurring at codon 12 and consisting predominantly of G to T transversions [5–12]. On the other hand, some studies have showed that K-*ras* mutations are found in only 5–7% of lung tumors from nonsmoking lung cancer patients [6,8,10,12]. Taken together, these results suggested that K-*ras* mutations were induced primarily by tobacco-smoke carcinogens. It has been suggested that such mutations occur in carcinogen-exposed cells during the misreplication of a bulky DNA adducts formed between a carcinogen(s), such as PAHs found in tobacco smoke, and guanine bases in the gene's sequence [13,14].

We previously showed that lung tumors from a group of 24 nonsmoking women exposed to smoky coal emissions from XW contained a high frequency of *KRAS* and *TP53* mutations [14]. Here, we expand this study to analyze K-*ras* mutations in specimens from additional 102 lung cancer patients from XW: 41 nonsmokers and 61 smokers, and 14 nonsmoking women and 36 men (mostly smokers) from Beijing and Henan (B&H), China, where natural gas is the main fuel. This has permitted us to compare the K-*ras* mutation frequency and mutation spectrum in a larger population of nonsmoking women exposed to smoky coal and Chinese populations who were not exposed to these emissions.

2. Subjects and methods

2.1. Patients, lung tumor tissues and sputum samples

XW lung cancer patients who donated sputum samples in this study were part of the lung cancer cases that were initially involved in a previous study [15]. These lung cancer cases were diagnosed based on a minimum of clinical symptoms and chest X-ray analysis at the XW Hospital. A total of 22 surgically removed fresh-frozen or paraffin-embedded lung tumors were used in this study. The clinical and demographic information of patients (histologic type of tumor, gender, age, fuel use history, tobacco smoke history, diet and occupation) was obtained from case history records from XW hospital. In addition, sputum samples collected from 80 lung cancer patients identified at XW hospital were analyzed. For these patients, a

standardized closed questionnaire was used to obtain demographic information, smoking history, family and personal medical history, as well as information on other variables [15]. However, the information on the histologic types of lung cancer could not be obtained from many of these patients from XW Hospital. Most of these individuals did not undergo surgical removal of lung tumor and were able to provide only sputum samples. The comparison group of patients was from Beijing and Zhengzhou, the capital city of Henan Province, where natural gas is mainly used in homes. Surgically removed tumor specimens were obtained from 20 lung cancer patients from Cancer Hospital, Chinese Academy of Medical Science and Beijing Friendship Hospital, and sputum samples were obtained from 30 lung cancer patients from the Hospital of Henan Medical University located in Zhengzhou. The pathological diagnosis, using established morphologic criteria, was available for these patients (Table 1). For the protection of human subjects, this study was conducted according to recommendations of the World Medical Association Declaration of Helsinki (1989) [16]. The study subjects provided informed consent to participate in this study. The research protocol met the requirements for protection of human subject certification by the US EPA.

Table 1 Patients distribution, demographic and clinical information

	Xuan Wei	Beijing and Henan
Age (mean, range)	52 ± 12 ($n = 90$)[a]	54 ± 12 ($n = 50$)
Female (age)	52 ± 11	51 ± 12
Male (age)	51 ± 13	55 ± 11
Sex N (%)		
Females	41 (40.2)	14 (28.0)
Males	61 (59.8)	36 (72.0)
Smoking status (%)[b]		
Nonsmokers	41 (40.2)	17 (34.0)
Smokers	61 (59.8)	33 (66.0)
Histologic tumor types[c]		
Adenocarcinoma	18	17
Adenosquamous	1	0
Squamous cell carcinoma	2	26
Small cell carcinoma	1	7

[a] The total number of patients was 102, no age information was available for 12 patients.

[b] In Xuan Wei, all 41 nonsmokers were females while all 61 smokers were males. In Beijing and Henan, all 33 smokers were males while the 17 nonsmokers included 14 females and three males.

[c] The histologic types of lung cancer in Xuan Wei were calculated based on the information from tumors only ($n = 22$).

First-morning sputum samples were collected on five consecutive mornings from all study subjects. All subjects rinsed their mouth with water to remove extraneous material. Subjects were then instructed to take a deep breath, cough deeply and expectorate into a plastic cup. Each morning sputum sample was stored in 40 ml of Saccomanno's fluid (39% ethanol, 3% polyoxyethylene, and 2% isopropanol; Lerner Laboratories, Pittsburgh, PA) to fix and preserve the cells. The sputum samples were stored at 4 °C and transported to the US by air. To collect cells, each sputum sample in Saccomanno's fluid was blended for 8–15 s in a blender to break the mucus and free the cells. The sample was then centrifuged at 600 × *g* for 10 min. The supernatant was discarded and the cell pellet was resuspended in fresh Saccomanno's fluid by vortexing to achieve a cell concentration of approximately 10^6 cells per ml. The cells were subjected to cytological examination using the method described by Saccomanno in order to determine whether the sputum samples were derived from the lower respiratory tract and also to confirm the presence of tumors and atypical cells in sputum samples [15].

2.2. DNA extraction and analysis of K-*ras* mutations

For DNA isolation, each fresh-frozen lung tissue section was dissociated in a lysis buffer (10 mM Tris, pH 7.4, 0.5% SDS, 150 mM NaCl, 100 mM EDTA), and digested with RNase A1 (10 mg/ml, at 37 °C for 2 h) and proteinase K (20 mg/ml at 37 °C for at least 4 h). DNA was recovered by phenol–chloroform extraction, and ethanol precipitation. Paraffin-embedded tissue sections were individually deparaffinized in xylene, ethanol, and rinsed with water. DNA was extracted using the same DNA extraction method used for fresh-frozen tissues. For mutation analysis, exon 1 of the K-*ras* gene was amplified by PCR using 50–100 ng of DNA extracted from each tumor and analyzed by denaturing gradient gel electrophoresis (DGGE), as described previously [10]. For DNA extraction from sputum samples, an aliquot from each specimen in Saccomanno's fluid containing approximately 2×10^4 cells was centrifuged. The cell pellet was washed twice with phosphate buffered saline, resuspended in a lysis buffer and DNA was extracted using RNase A1/proteinase-K digestion, phenol–chloroform extraction, and ethanol precipitation. An Aliquot

containing an equivalence of about 5×10^3 cells was used for analysis of mutations in codon 12 of the K-*ras* gene using a sensitive method combining a mutant allele enrichment (MAE) and DGGE, as well as the reagents and conditions described previously [17].

2.3. Statistical analysis

The types and distribution of K-*ras* mutation among males and females from each group were analyzed for statistical significance using Fisher's Exact Test, using program STATA.

3. Results

In this study, K-*ras* mutations were analyzed in specimens, either lung tumors or sputum samples, obtained from 152 lung cancer patients from China (see Table 1). This includes 102 patients (41 females and 61 males) from XW where smoky coal was used for domestic fuel and the lung cancer mortality rate was high [2]. All XW female patients were nonsmokers and had an average age at diagnosis of 52 ± 11 years (28–58 years), while all male patients were smokers and had an average age at diagnosis of 51 ± 13 years (28–60 years). In addition, 30 patients (seven females and 23 males) from Henan, and 20 patients (seven females and 13 males) from Beijing, were analyzed as a comparison group. In these regions, the residents were not exposed to unvented smoky coal combustion emissions and the lung cancer rate approximated the average rate for China [2]. All 14 female patients from Henan and Beijing were nonsmokers and had an average age at diagnosis of 51 ± 12 years (28–64 years). The male patients were mostly smokers (33 of 36) and had an average age at diagnosis of 55 ± 11 years (27–74 years).

The results of K-*ras* mutations found in lung tumors or sputum samples obtained from the 152 lung cancer patients are summarized in Table 2. Among the 102 patients from XW, nine of 41 females (21.9%) had a K-*ras* mutation in their lung tumor or sputum sample, including four GTT (valine), two TGT (cysteine), two GAT (aspartate), and one CGT (arginine) (the wild type codon 12 is GGT or glycine). Therefore, G to T transversions account for 66.7% of all K-*ras* mutations found in specimens from female lung cancer patients from XW. Among the 61 males, 14 (22.9%) had a K-*ras* mutation in their lung tumor or sputum sample. These included nine TGT (cysteine), three GTT (valine), one CGT (arginine), and one GCT (alanine). Therefore, 85.7% of the K-*ras* mutations found in male lung cancer patients in XW are G to T transversions. Altogether, K-*ras* mutation frequencies are similar between nonsmoking women and cigarette smoking men in XW (21.9 vs. 22.9%, respectively; $P = 1.00$, two-sided Fisher's Exact Test). Also, the G to T transversions predominated among the mutations found in both the female and male patients (66.7 vs. 85.7%, respectively). Among the 50 lung cancer patients from B&H, seven K-*ras* mutations were detected. One (7.1%) mutation, a TGT (cysteine), was found among 14 female patients. Among the 36 males, six (16.7%) mutations, including three GTT (valine), two GAT (aspartate), and one TGT (cysteine), were identified and included 66.7% G to T transversions. Therefore, in B&H, the K-*ras* mutation frequency in nonsmoking women is lower than, although not statistically significant from, that of smoking men (7.1 vs. 18.2%, respectively; $P = 0.657$, two-sided Fisher's Exact Test) because of the small number of samples.

Table 2 Types and distribution of K-*ras* mutation among female and male patients

Mutation[a]	Xuan Wei	Beijing and Henan
Females	41	14
CGT (Arginine)	1	0
GAT (Aspartate)	2	0
GCT (Alanine)	0	0
GTT (Valine)	4	0
TGT (Cysteine)	2	1
Total	9 (21.9%)[c]	1 (7.1%)[d,e]
Males[b]	61	36
CGT (Arginine)	1	0
GAT (Aspartate)	0	2
GCT (Alanine)	1	0
GTT (Valine)	3	3
TGT (Cysteine)	9	1
Total[b]	14 (22.9%)	6 (18.2%)

[a] The wild type K-ras codon 12 is GGT (glycine).
[b] The mutant frequencies are calculated for smoking men. In B&H, three of the 36 men were nonsmokers.
[c] 21.9 vs. 22.9%, $P = 1.00$, two-sided Fisher's exact test.
[d] 7.1 vs. 18.2%, $P = 0.657$, two-sided Fisher's exact test.

4. Discussion

K-*ras* mutations are common and consist mostly of G to T transversions in lung tumors from smoking lung cancer patients [5–12] but are infrequent in

lung tumors from nonsmoking lung cancer patients [6,10–12]. These transversions were induced in cultured cells treated with tobacco smoke carcinogens, such as the polycyclic aromatic hydrocarbon benzo(a)pyrene [18,19]. These results suggested that K-*ras* mutations observed in lung tumors from smokers were associated with exposure to carcinogens in tobacco smoke. We here demonstrate that K-*ras* mutations are frequent (21.9%) and consist mostly of G to T (66.7%) in nonsmoking female lung cancer patients in XW. These results confirm our previous finding of a 29.2% (seven of 24 cases) frequency of K-*ras* mutations consisting predominantly of G to T transversions (85.7% or six of seven mutations) in 24 of these women whose lung tumors had been also analyzed for *TP53* mutations [14]. Our results showed that the frequency and type of K-*ras* mutations in nonsmoking women with lung cancer in XW are similar to those found in smoking male patients in XW and in B&H. They are also similar to those reported for smoking female and male lung cancer patients in other studies [5–12]. The average K-*ras* mutation frequency in XW women in this study and our previous study [14] is 24.6% (16 mutations among 65 women). It is higher than the 7.1% frequency observed in nonsmoking women in B&H, although the difference is not statistically significant due to the small number of the B&H women ($P = 0.28$, two-sided Fisher's Exact Test). On the other hand, the 24.6% mutation frequency is significantly different from the 8% (four mutations among 50 women) mutation frequency determined for nonsmoking women from both B&H in this study and from the Western Pennsylvania region in our previous study ($P = 0.025$, two-sided Fisher's Exact Test) [10,12]. It is also significantly different from the 5% frequency (two mutations among 40 patients) for nonsmoking female and male lung cancer patients from Europe ($P = 0.015$, two-sided Fisher's Exact Test) [6]. These results suggest an association between the increased K-*ras* mutation frequency in lung cancer in nonsmoking women and exposure to coal combustion emissions in XW.

Specimens from lung cancer patients analyzed in this study consisted of either lung tumor tissues or sputum samples. Previously, we have applied our sensitive PCR + MAE method to compare K-*ras* mutations in lung tumors and matched sputum samples obtained from lung cancer patients from the University of Pittsburgh Medical Center [20]. K-*ras* mutations were detected in sputum samples of lung cancer patients with both adenocarcinomas and squamous cell carcinomas of all tumor grades, and in the presence or absence of lymph node metastasis. We have demonstrated a highly significant association between the detection of a K-*ras* mutation in sputum samples and the presence of the identical mutation in the matched tumor sample (Kappa = 0.64, percent confidence interval 0.32–0.95, $P < 0.01$). These data suggested that a sensitive detection of K-*ras* mutations in sputum samples provide a useful diagnostic marker for lung cancer. Therefore, the difference of mutation frequencies observed between XW women and other groups of lung cancer patients should not be associated with the use of lung tumors and sputum samples as biological specimens in this study.

Another factor that may affect K-*ras* mutation frequencies is the variation in the proportion of adenocarcinomas and squamous cell carcinomas, the two most frequent histologic types of lung tumors, observed among the various groups of lung cancer patients. K-*ras* mutations may occur more frequently in one type of lung tumor than the other. For instance, our previous studies showed that K-*ras* mutations were more frequent in adenocarcinoma than squamous cell carcinoma in the Western Pennsylvania region [10]. Based on the information available for the tumor types from nine women and 13 men who had their lung tumor surgically resected in XW, adenocarcinomas were more frequent than squamous cell carcinomas in both women (77.8 vs. 11.1%) and men (84.6 vs. 15.4%). In B&H, on the other hand, the proportions of adenocarcinomas relative to those of squamous cell carcinomas are similar in women (42.8 vs. 42.8%) and even lower in men (30.6 vs. 55.6%). Nevertheless, K-*ras* mutations were detected at comparable frequencies between lung adenocarcinomas and lung squamous cell carcinomas. For instance, 18 of 22 (81.8%) tumors from XW were adenocarcinomas (Table 1) while seven of the eight (87.5%) mutations were found in these tumor types. Likewise, 26 of 50 (52%) tumors from B&H were squamous cell carcinomas with five of the seven (71.4%) mutations found in these tumor types. These results showed that K-*ras* mutations were frequently found in both adenocarcinomas and squamous cell carcinomas of the lung in lung cancer patients from China. They suggest that the difference in K-*ras* mutation frequencies observed between the XW women and B&H women is more likely associated with exposure to smoky coal combustion emissions than with a variation in major histologic types of lung tumors in these women.

With regard to the potential etiologic factors inducing such mutations, although these women were mostly exposed at home to indoor coal combustion emissions in XW, one must take into

account the fact that most of them lived with smokers and, therefore, were exposed also to environmental tobacco smoke. However, the extent of such an exposure should be much less than that in men because the urine cotinine levels in these women represented only 2% of those found in men [21]. Likewise, male lung cancer patients in XW were not only smokers but also were exposed at home to indoor coal combustion emissions. Therefore, both exposure to coal combustion emissions and tobacco smoke should be considered as potential etiologic factors that may induce K-*ras* mutations in XW lung cancer. We do not have female smokers or male nonsmokers among the lung cancer patients in XW. Information on K-*ras* mutation frequencies among such groups of patients may help us understand whether exposure to both smoky coal combustion emissions and tobacco smoke could have any additive effect on K-*ras* mutation frequencies in lung cancer patients in XW. Nevertheless, in comparison with B&H, where residents were not exposed to unvented smoky coal combustion, the lung cancer rates among women in these regions were still much lower than those in XW women. This suggests that the increased rate of lung cancer among women in XW is associated with indoor exposure to coal combustion emissions. The rates of lung cancer among male smokers in XW and in B&H areas are not significantly different although those in XW were exposed also to coal smoke, suggesting that lung cancer observed in male smokers in XW was associated mainly with tobacco smoke exposure. Taken together, these results suggest that K-*ras* mutations observed in XW women are induced by an exposure to lung carcinogen(s) found in coal combustion emissions. Furthermore, the close similarity between the frequencies and types of K-*ras* mutations in these women and those observed in male smokers in this County suggest that such carcinogen(s) found in coal combustion may be similar to those found in tobacco smoke.

Previous studies showed that organic compounds extracted from smoky coal combustion emissions produced an increased number of transformed cells in vitro and were mutagenic in the Ames test [22,23]. The most active fraction of these compounds contained mainly PAHs and alkylated PAHs that were shown to be potent carcinogens in mouse skin tumorigenicity assays [24–26]. PAH-DNA adducts have been found in white blood cells, placenta, and bronchoalveolar lavage obtained from individuals exposed to indoor air pollution in XW [27,28]. Therefore, K-*ras* mutations found in lung tumors and sputum samples from nonsmoking female lung cancer patients from XW may be induced by carcinogens found in coal combustion emissions, such as the PAHs and PAH-derived compounds.

In summary, in this study we demonstrate that K-*ras* mutations were frequently found in lung tumors and sputum samples obtained from nonsmoking female lung cancer patients from XW. Both the frequencies and types of these mutations were similar to those of K-*ras* mutations found among smoking male lung cancer patients from both XW and B&H. These results suggest a similarity of origin(s) and/or mechanisms of formation of K-*ras* mutations in nonsmoking women and in cigarette smoking men in XW. These mutations may be induced by PAHs and/or PAH-derived compounds found in both coal combustion emissions and tobacco smoke.

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